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Running head: Developmental abnormalities produced by naphthenic acids

Title: **Predicted and measured acute toxicity and developmental abnormalities in zebrafish embryos produced by exposure to individual aromatic acids**

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Highlights [AS1]

- Acute toxicity of 16 individual aromatic ‘naphthenic’ acids assayed (invertebrate).
- Lethality and developmental abnormalities of 6 individual acids assayed (fish).
- Toxicities modelled using commercially-available Admet predictor™ software.
- Significant relationship found between predicted and experimental fish LC_{50}s.
- Embryo abnormality EC_{50}s <7μM in 4 of 6 acids; 4-(p-tolyl)benzoic acid most toxic.

37

38 Abstract

39 Petroleum acids, often called ‘Naphthenic Acids’ (NA), enter the environment in
40 complex mixtures from numerous sources. These include from Produced and
41 Process-Affected waters discharged from some oil industry activities, and from the
42 environmental weathering of spilled crude oil hydrocarbons. Here, we test the
43 hypothesis that individual NA within the complex mixtures can induce developmental
44 abnormalities in fish, by screening a range of individual acids, with known chemical
45 structures.

46 Sixteen aromatic NA were tested using a *Thamnocephalus platyrus* (beavertail
47 fairyshrimp) assay, to establish acute toxicity. Toxicities ranged from 568 to 8 μM,
48 with the methylbiphenyl acid, 4-(*p*-tolyl)benzoic acid, most toxic.

49 Next, five of the most toxic monoacids and, for comparison, a diacid were assayed
50 using *Danio reri*o (zebrafish) embryos to test for lethality and developmental
51 abnormalities. The toxicities were also predicted using Admet predictor™ software.
52 Exposure to the five monoacids produced deformities in zebrafish embryos in a
53 dose-dependent manner. Thus, exposure to 4-(*p*-tolyl)benzoic acid produced
54 abnormalities in >90% of the embryos at concentrations of <1μM; exposure to
55 dehydroabietic acid caused pericardial edema and stunted growth in 100% of the
56 embryos at 6 μM and exposure to pyrene-1-carboxylic acid caused 80% of embryos
57 to be affected at 3 μM.

58 The findings of this preliminary study therefore suggest that some aromatic acids are
59 targets for more detailed mechanistic studies of mode of action. The results should
60 help to focus on those NA, which may be important for monitoring in oil industry
61 wastewaters and polluted environmental samples.

1. Introduction

Crude oil and its refined fractions make major contributions to contamination of some aquatic environments, especially in the vicinities of urban and industrial areas (NRC, 2003; GESAMP, 2007; Jernelov, 2010; White et al., 2013). However, since some deleterious effects on aquatic organisms could not be explained by exposure to the most abundant petroleum contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and alkyl PAHs, studies have also focused occasionally on more 'polar' compounds (e.g. Knag et al., 2013). The so-called 'naphthenic acids' (NA) are often the most abundant polar compounds in petroleum (e.g. Melbye et al., 2009; Thomas et al., 2009; Knag et al., 2013; Ruddy et al., 2014). The term NA describes broadly those carboxylic acids in biotransformed petroleum, including in oil sands and in biodegraded, spilled and reservoired, crude oils (Clemente and Fedorak, 2005; West et al., 2014; Brown and Ulrich, 2015; Headley et al., 2015). Following releases of crude oil into the environment, NA and other polar components may be detected, either because they were already present, or because of subsequent physico-chemical 'weathering' including photo-oxidation and/or microbiological oxidation. For instance, after the Deepwater Horizon, Macondo well spill in 2010 released an estimated 4.9 million barrels of crude oil (780000 m^3) into the Gulf of Mexico; analyses of beached oil revealed a diverse range of polar compounds, including NA (Ruddy et al., 2014). The undegraded spilled Macondo well oil already comprised ~10% polar constituents (Reddy et al., 2012) and therefore around 80000 m^3 of these substances were possibly released directly into the environment initially and this was likely increased by subsequent natural transformations of the hydrocarbons (Aeppli et al., 2012). Indeed, NA metabolites for anaerobic oxidation of the Macondo well oil were reported in Gulf of Mexico sediments close to Deepwater Horizon

following the spill (Kimes et al., 2013; Valentine et al., 2014). Wan et al., (2014) also measured increased concentrations of NA in sediments following the Hebei Spirit oil spill.

In addition to microbial processes, in which NA are produced from the hydrocarbons either in-reservoir or in the environment (e.g. reviewed by Agrawal and Gieg, 2013), hydrocarbon metabolism by sediment-dwelling organisms such as worms also produces the corresponding acids (Malmquist et al., 2015). Thus, there are a number of pathways by which NA may enter the environment and by which organisms may become exposed to petroleum-derived NA.

Various toxic effects have been attributed to NA (reviewed by Clemente and Fedorak, 2005; Kannel and Gan, 2012; Brown and Ulrich, 2015; Mahaffey and Dube, 2017). Several individual NA were tested using the bioluminescence inhibition assay Microtox™ and *Daphnia magna* acute lethality test; toxicities were found to be in the range 0.01 – 29 mM (Frank et al., 2009). Jones et al., (2011) tested the acute toxicities of a range of individual NA using Microtox™ and reported that only a few NA had EC_{50} values <50 μM. Interestingly, the concentration curves reported by Jones et al., (2011) were very steep, a phenomenon also reported for exposure to NA fractions when tested on larvae of zebrafish, prompting the hypothesis that the surfactant properties of NA were having a significant influence on toxicity (Scarlett et al., 2013). Tollefsen et al., (2012) tested the toxicity of some synthetic NA to rainbow trout liver cells. The combined toxicity of multicomponent mixtures of the NA was also assessed using the concept of concentration addition and independent action prediction. All of the acids tested had EC_{50} values in the range 108–405 μM (24–89 mg L^{-1}) and 188–656 μM (43–148 mg L^{-1}) when assessed by effects on metabolic inhibition or loss of membrane integrity, respectively. Binary and 6-compound

112 mixtures of NA caused combined toxicity according to the concept of additivity,
113 although slight deviations from additivity were observed at a few mixture
114 concentrations. More recently, Petersen et al., (2017) reported the results from *in*
115 *vitro* screening on fish cells, which indicated that of the endpoints tested, the
116 predominant toxic mode of action (MoA) was cytotoxicity. EC_{50} values for cytotoxicity
117 were obtained for 16 compounds and ranged from 77 µM–24 mM, whereof aliphatic
118 monocyclic acids, monoaromatic acids, polycyclic monoaromatic acids were
119 amongst the most toxic. The observed cytotoxicity of the chemicals correlated well
120 with the hydrophobicity (Log K_{OW}) suggesting that the toxicity was predominantly due
121 to a non-specific MoA. Scarlett et al., (2012) studied the estrogenic and androgenic
122 activity of individual adamantane acids using a panel of human cell-derived nuclear
123 receptor reporter gene bioassays (CALUX®) but found no significant activity.
124 However, genotoxic activity was observed in hepatocytes of marine mussels
125 following *in vivo* exposures to adamantane carboxylic acids (Dissanayake et al.,
126 2016).

127 Toxicity models (ADMET predictor™, Lancaster, CA, USA) have also been applied
128 to prediction of the toxicities of individual NA and thus far, at least, have generally
129 been in good agreement with results obtained from the small number of assays
130 made on individual NA (Scarlett et al., 2012).

131 The early life stages (ELS) of fish appear to be particularly sensitive to some
132 petroleum components (e.g. Incardona, 2017; Sorensen et al., 2017)and some
133 studies strongly implicate NA as causative agents for developmental abnormalities in
134 fish. For example, Marentette et al., (2015a) reported that exposure of fathead
135 minnow *Pimephales promelas* embryos to NA fractions derived from fresh and aged
136 oil sands process-affected water (OSPW) produced significant dose-response

relationships of cardiovascular edemas and/or haemorrhages. Exposure to commercially-available NA mixtures, did not result in such relationships, but did produce an increase in finfold deformities (Marentette et al., 2015b). This implies that development of abnormalities in fish embryos may be dependent on particular NA. Reinardy et al., (2013) noted that a fraction of NA classified as broadly alicyclic, produced no estrogenic response in embryonic fish, whilst an 'aromatic' NA fraction isolated by argentation chromatography, did produce a (albeit weak) response.

Knowledge of the toxic effects of individual NA is therefore still extremely limited. Until relatively recently, even methods for the identification of the individual acids in the complex NA mixtures from petroleum and oil sands, did not exist. However, some acids have now been identified (Rowland et al., 2011a; Rowland et al., 2011b; Rowland et al., 2011c; Jones et al., 2012; Lengger et al., 2013; Bowman et al., 2014; Wilde and Rowland, 2015; Wilde et al., 2015; Wilde and Rowland, 2018).

Given the paucity of studies of the toxic effects of individual NA and the observations that fish exposed to NA fractions derived from OSPW suffered developmental abnormalities (Marentette et al., 2015a), the present preliminary study was designed to test the hypothesis that exposure to individual aromatic acids might result in developmental abnormalities consistent with those observed for more complex mixtures of NA. A range of aromatic acids found previously to be associated with OSPW and in biodegraded crude oils and petroleum-contaminated aquifers (Annweiler et al., 2001; Elshahed et al., 2001; Boll et al., 2002; Gieg and Suflita, 2002; Aitken et al., 2004; Safinowski et al., 2006), or as the products of PAH biotransformation (e.g. Malmquist et al., 2015), were screened. The chemicals were chosen so that greater insight could be gained into any structural activity relationships. In particular, the hypothesis that toxicity might be independent of

structure and similar for isomeric chemicals with identical elemental formulae was tested. The results were compared to modelled data for the waterflea *D. magna* and fathead minnow *P. prome*las to examine whether prediction software, which takes account of the physicochemical properties, could predict toxicities more accurately. Zebrafish embryos were then exposed to a subset of six of these chemicals; 96h LC_{50} values were calculated and any deformities recorded and used to calculate 96h EC_{50} values.

2. Materials and Methods

2.1 Selection of test chemicals

Sixteen [SR2][AS3]acids were chosen for initial lethality screening (Table 1). All test chemicals were purchased from Sigma-Aldrich (Poole, UK) with the exception of Dehydroabietic acid (DHAA, Orchid Cellmark, New Westminster BC, Canada) and had minimum stated purities ≥97%. Thamnotoxkit FTM was from MicroBioTests Inc. (Gent, Belgium).

2.2 Preparation of test solutions

The preparation of test solutions was similar to that previously reported for zebrafish larvae exposures to NA (Reinardy et al., 2013; Scarlett et al., 2013). Stock solutions of test chemicals were made prior to the exposure in amber glass vials by diluting in 1 M NaOH until fully dissolved and a known volume of exposure water. The pH of the stock was then adjusted to pH 7.4 ± 0.1 using 1 M HCl [AS4]and stored at 4 ºC in amber vials until use. A negative control solution was prepared comprising of the

highest NaOH concentration used and pH adjusted to match test solutions. Compared to water controls, no significant effects were observed following exposure to this NaOH control solution and all test results presented are relative to this. The concentration range tested for each chemical was based on the predicted lethality of *D. magna* and *P. promelas* (ADMET predictor™, Lancaster, CA, USA).

2.3 Fairyshrimp acute lethality assay

The acute toxicity of the test materials was evaluated using the freshwater beavertail fairyshrimp. Exposure water was made as specified by ISO 7346-1 (ISO, 1996). The test was performed according to manufacturer's instructions (available online: http://www.microbiotests.be/SOPs/Thamnotoxkit%20F%20SOP%20-%20A5.pdf accessed 25.01.2018). Briefly, the test 24 h LC_{50} test was performed in a 24-well test plate using instars II–III larvae of the shrimp, which were hatched from cysts. Hatching was initiated 24 h prior to the start of the test. Upon hatching, 10 shrimp were exposed to various concentrations (n = 3) of the compounds and incubated at 26°C for 24 h in the dark. The test endpoint was mortality. The numbers of dead shrimps for each concentration were recorded and the respective LC_{50} values [AS5]were determined using Probit or Logit analysis in SPSS 21.

2.4 Zebrafish embryo test

Wild-type WIK (Wild-Type India Calcutta) strain zebrafish embryos were obtained from the Max Plank Institute, Tubingen, Germany, and maintained at the University of Exeter as described in the supplementary material (SI).

208 The test was initiated as soon as possible after fertilization of the embryos and not
209 later than 3 h post-fertilization (128-cell stage). The exposures used 300 mL
210 crystalline dish test vessels with 100 mL of test solutions containing 20 embryos with
211 3 replicates per concentration and employed a semi-static renewal technique.
212 Temperature was maintained at 26±1 °C, and fish were kept under a constant
213 artificial dark/light cycle of 8/16 h. [AS6]Every 24 hrs, up to four apical observations
214 were recorded as indicators of lethality (i) coagulation of fertilised eggs, (ii) lack of
215 somite formation, (iii) lack of detachment of the tail-bud from the yolk sac, and (iv)
216 lack of heartbeat. At the end of the 96 h exposure period, acute toxicity was
217 determined based on a positive outcome in any of the four apical observations
218 recorded, and LC_{50} calculated. Embryos were exposed for 96 h, with viability being
219 checked every 24 h and dead embryos removed.

220 The results were deemed acceptable if: fertilisation rate of the eggs ≥70%, dissolved
221 O_2 concentration in the negative control and highest test concentration ≥80 % of
222 saturation at test commencement, water temperature maintained at 26±1 °C in test
223 chambers at all times, overall survival of embryos in the negative/NaOH control ≥90%
224 and hatching rate in the negative control ≥80 % at test termination. The number of
225 dead fish and abnormally developed embryos for each concentration were recorded
226 and the respective LC_{50} and half-maximal effect (EC_{50}) determined using Probit or
227 Logit analysis in SPSS 21 based on Pearson Goodness-of-Fit test.

228

229 *2.5 Predictive toxicity*

230 Commercially available software, ADMET predictor™ (Simulations Plus Inc.,
231 Lancaster, CA) was used to predict physiochemical properties of the test chemicals

232 plus lethal and sublethal toxicities. ADMET predictor™ uses chemical structures and
233 experimental data to create the QSAR models which are then used to predict
234 properties of the molecules. The models provide a range of physicochemical outputs,
235 including log P (i.e. predicted log Kow) and water solubility provided in Table 1, plus
236 predictive toxicity to organisms including *D. magna* (48 h exposure lethality (LC_{50})
237 and *P. promelas* (96 h exposure lethality (LC_{50}). Predictive models that generate
238 EC_{50} values for developmental abnormalities were not available but a model that
239 predicts a binary yes/no outcome for reproductive/ developmental toxicity that relates
240 to anything that disturbs the reproductive process of organisms, including adverse
241 effects to sexual organs, behaviour, ease of conception, and developmental toxicity
242 of offspring both before and after birth was available (ADMET predictor™). Full detail
243 of the model's training/verification sets are available online http://www.simulations-
244 plus.com/software/admetpredictor/toxicity/, accessed 25.01.2018. The LC_{50} values used
245 to build the models were for species not routinely tested in our labs. As this is also
246 likely for many other lab studies we wished to test if, despite these differences, there
247 was reasonable agreement between experimental and predicted toxicities. If so,
248 there can be greater confidence in the predictions as they will not be specific to a
249 certain species. Regression analyses for experimental versus predicted LC_{50} values
250 were calculated using Statgraphics Online (Warrenton, Virginia, US).

251

252 **3. Results**

253 *3.1 Acute lethal toxicity to fairyshrimp*

254 A large range of acute LC_{50} values was observed for the chemicals tested (Table 2).
255 4-(p-tolyl)benzoic acid (A10; Table 2), which has a biphenyl structure, was the most

toxic NA with a LC_{50} of 8 μM. Phenanthrene-4-carboxylic acid was the least toxic with a LC_{50} of 568 μM. Comparing toxicities of NA with the same chemical formula, some, such as the tetralin acids (tetralin 1- and 5-carboxylic acids, A2 and A3; $C_{11}H_{12}O_2$) had reasonably similar LC_{50} values (i.e. within a factor of two), whilst others were very different (e.g. one of the diaromatic acids 4-(p-tolyl)benzoic acid (A10; $C_{14}H_{12}O_2$) was about 30× more toxic than another (2-benzylbenzoic acid, A11; Table 2). Comparing the experimental and predicted toxicities, the experimental data obtained for fairyshrimp were generally in reasonable agreement with the predicted LC_{50} values (albeit for another aquatic crustacean species, the waterflea *D. magna*; Table 2). Five of the chemicals had LC_{50}s less than those predicted by a factor of 2, but all were within an order of magnitude. However, linear regression of experimental versus predicted LC_{50} values produced an r^2 value of 0.066 which was not statistically significant (p >0.05).

3.2 Acute lethal toxicity to zebrafish

Concentration response curves for exposures of zebrafish were generally very steep for the five chemicals for which LC_{50} values could be determined (Fig. 1). A value could not be generated for naphthalene-2,6-dicarboxylic acid as no mortality occurred. For the exposures to acids for which LC_{50} values were generated, the values ranged from 4.9 μM for 4-(p-tolyl)benzoic acid (A10;Table 2) to 151μM for tetralin-5-carboxylic acid (A3; Table 2). Pyrene-1-carboxylic acid (A 15) and DHAA (A16) had LC_{50} values of 7-8 μM. Experimental LC_{50} values for zebrafish were mainly within an order of magnitude of the predicted values for fathead minnow, despite the fact that the model was based on a different fish species and was not

specific to early life stages (Table 2). Linear regression produced the relationship: experimental LC_{50} (μM) = 0.302 × predicted LC_{50} (μM) - 5.4 (r^2 = 0.934, p < 0.01, n = 5).

3.3 Developmental abnormalities in zebrafish

Abnormalities (>10%) in zebrafish embryos were observed in five of the six chemicals tested. Deformities were no greater than controls in naphthalene-2,6-dicarboxylic acid, but exposure to all of the monoacids produced concentration-dependent abnormalities including yolk-sac and pericardial edema, fin abnormalities, tail flexure and truncation, and reduced growth (illustrated for pyrene-1-carboxylic acid, A15 in [SR7][AS8]Fig. 2). In addition, head and ocular edema were also observed in embryos exposed to naphthalene-2-carboxylic acid (A1; Tables 1 & 2), but not for exposures to the other chemicals (Fig. 3). Effects were only observed at relatively high concentrations for some compounds (e.g. tetralin-5-carboxylic acid, A3) produced about 80% deformities, mainly yolk-sac edema, at 96 hours post fertilisation (hpf) at 32 μM (Fig S1) produced an EC_{50} of 32 μM (Table 2). However, abnormalities were observed earlier and at much lower concentrations, for other acids. For example, following exposure to pyrene-1-carboxylic acid (A15), around 80% of embryos were affected at 3 μM and virtually all by 6 μM at 72 hpf (Fig. 2; Table 2). The diaromatic NA 4-(p-tolyl)benzoic acid (A10), produced around 90% deformities at <1 μM with an EC_{50} of 0.8 μM (Table 2). Abnormalities were predominantly pericardial edema and were observed at 24 hpf (Table 2; Fig S2). DHAA produced the same range of deformities in 100% of embryos at a concentration of 6 μM at 96 hpf (Fig. S3). Reproductive/developmental toxicity was predicted for two of the six

304 chemicals, but developmental abnormalities in embryos were observed for only one
305 of these: pyrene-1-carboxylic acid (A15). This tetra-aromatic acid produced an EC_{50}
306 of 2.3 μM (Table 2). Four chemicals predicted not to cause
307 reproductive/developmental toxicity did produce abnormalities (Table 2).

308

309 **4. Discussion**

310 *4.1. Fairyshrimp screening assay*

311 The acids tested in the initial screening assay (Table 1) were chosen on the basis of
312 their occurrence in various petroleum-related scenarios. Thus, naphthoic-type acids
313 (A1,4,5,12; Table 1) are known biodegradation products of naphthalene and alkyl
314 naphthalenes in petroleum (e.g. reviewed by West et al., 2014) and tetralin
315 carboxylic acids (A2,3; Table 1) are known constituents of OSPW (Bowman et al.,
316 2014). Phenanthrene (A13; Table 1) and pyrene (A15; Table 1) carboxylic acids are
317 known products of petroleum or alkylpyrene biotransformation (e.g. Malmquist et al.,
318 2013; Aitken et al., 2018). DHAA and isomeric acids have been reported in OSPW
319 (Jones et al., 2012) and toxicity data to fish exists so this acid also acts as a useful
320 cross reference for comparison with data from previous studies and as a check on
321 the validity of the present methods (Straus et al., 1997; Jones et al., 2012; Scarlett et
322 al., 2013). A diacid, naphthalene-2,6-dicarboxylic acid, was included as diacids are
323 known products of anaerobic biodegradation of petroleum hydrocarbons (e.g.
324 Agrawal and Gieg, 2013; Kimes et al., 2013). In order to better understand the
325 importance of chemical structure with regard to NA toxicity, this study included tests
326 on several pairs of chemicals with the same empirical formulae (Table 1).

For the tetralin acids (A2, 3; $C_{11}H_{12}O_2$) there was relatively little difference in toxicity (factor ~2) regardless of whether the carboxylic acid group was on the aromatic or cyclohexyl ring. Also, very similar acute toxicities to fairyshrimp were observed for two compounds with the formula $C_{13}H_{10}O_2$ (A6,7; Table 2).

The naphthoic acids (A1, 4, 5), which possessed either a methyl group or an ethanoic (acetic) acid group, again showed similar LC_{50} values (i.e. within a factor of ~2; Table 2). These examples would imply that chemical formula alone was a reasonable predictor of toxicity in such cases.

However, other NA with identical formulae showed very different toxicities. One of the diaromatic acids (4-(p-tolylbenzoic acid A10; $C_{14}H_{12}O_2$) was about 30× more toxic than another (2-benzylbenzoic acid, A11; Table 2). Clearly, knowledge of exact chemical structure is required to assess the potential toxicity of such NA. This is particularly important for monitoring NA in the environment.

4.2 Predictive models

The use of predictive models of toxicity offers a means of assessing the toxicities of specific chemicals, but is of most value if the numbers of false negatives and false positives, are acceptable. Pyrene-1-carboxylic acid (A15) was considerably more toxic than predicted (Table 2). On the other hand, there was a serious over estimation of the toxicity of phenanthrene-4-carboxylic acid (A13; Table 2). Carbazole-3-carboxylic acid (A9; Table 1) was included in the tests as nitrogen-containing acids of unknown structure have been reported in OSPW (e.g. Nyakas et al., 2013) and parent alkylcarbazoles are common environmental contaminants associated with diesel (Bennett and Larter, 2000). The LC_{50} was slightly below that predicted for the latter chemical (Table 2).

Experimental LC_{50} values for zebrafish showed generally good correlation with predicted values (for fathead minnow; Table 2; r^2 = 0.940; p < 0.01), although experimental values were ~3× lower (i.e. more toxic) than predicted. This is perhaps to be expected as embryos tend to be more sensitive than adults (e.g. Incardona, 2017; Sorensen et al., 2017) and the training set for the model was for fathead minnow rather than for zebrafish. Large scale multispecies studies of fish embryos and adults show good correlation in acute toxicity LC_{50} values, both between species and life stages, with embryos being most sensitive (Braunbeck et al., 2005). DHAA (A16, Table 1) was included in the study mainly for the purposes of providing a cross reference to other studies. The LC_{50} value of 7.7 µM DHAA for zebrafish embryos (Table 2) was similar to that reported previously (4 µM) for zebrafish larvae (Scarlett et al., 2013) and rainbow trout (Straus et al., 1997) and was also very close to the predicted LC_{50} of 5 µM (Table 2).

Taken as a whole, the use of the Admet predictor™ models[AS9] shows promise for the ability to correctly identify acutely toxic compounds. However, the occurrence of both serious over- and especially under-estimation of toxicity suggests that polar compounds are at present insufficiently represented in the training sets of the present models.

4.3 ~~Mode of toxic action~~[SR10] Potential mechanisms of toxicity

Both petroleum-derived NA mixtures and OSPWs containing NA, have been shown to cause a concentration-dependent increase in craniofacial and spinal deformities, reduced yolk utilization, time to hatch, hemorrhages, edemas and incomplete hatching in embryos of yellow perch (*Perca flavescens*) and Japanese medaka

(*Oryzias latipes*) (Peters et al., 2007). Exposure to OSPW was also found to produce increased rates of cardiovascular and spinal deformities, premature hatching and greater rates of spontaneous activity in embryos of fathead minnow (He et al., 2012). Such embryological effects are said to be associated with exposure to 'dioxin-like' compounds and mediated via the aryl hydrocarbon receptor (AhR) binding and CYP1A induction. He et al., (2012) found that the abundance of transcripts *cyp1a* was not significantly greater in embryos exposed to OSPW, so it was postulated that for NA, the effects may instead be due to increased oxidative stress and abnormally high rates of apoptosis (He et al., 2012). In another study, Marentette et al., (2015a) found that a fraction of OSPW containing NA also produced a range of deformities in embryos, thus strongly implicating NA as causative agents for producing developmental abnormalities in fish.

Our results also showed that some NA produce developmental abnormalities in fish embryos in a concentration-dependent manner (Fig. S1). However, an important difference is that our studies were made on individual acids (Tables 1, 2), not on complex NA mixtures (cf Peters et al., 2007; Marentette et al., 2015a; Marentette et al., 2015b). In this way the causative agents may be better constrained. Abnormalities (>10%) in zebrafish embryos were observed in five of the six chemicals tested i.e. all of the monoacids. Three acids were most toxic (A10, 15, 16). ~~Since the concentrations of the acids in the exposure waters nor the embryos was determined in this preliminary study, the toxic concentrations may arguably represent underestimates~~[AS11]~~.~~

The monoaromatic tricyclic DHAA (A16) produced a range of deformities in 100% of embryos at a concentration of 6 µM at 96 hpf (Fig. S3). Although DHAA has been linked to embryo toxicity (Sepulveda et al., 2003), no previous reports concerned

400 with the effects of DHAA on fish embryo development were found. DHAA is a
401 common contaminant in wood pulp and paper mill effluent (e.g. Rissanen et al.,
402 2003). ‘ The diaromatic, 4-(p-tolyl)benzoic acid (A10) produced around 90%
403 deformities at <1 µM and tetraaromatic, pyrene-1-carboxylic acid was found to
404 cause developmental abnormalities in 80% of embryos at 3 µM and virtually all by 6
405 µM at 72 hpf (Fig. 2). Although the present study showed that higher concentrations
406 of naphthalene-2-carboxylic acid (A1) were required to produce developmental
407 abnormalities, unusual head and ocular edemas were noted (Fig. 3).
408 Hydroxynaphthoic acid isomers have also been found to cause a range of
409 developmental abnormalities in fish embryos (*Oryzias latipes*), in the range 10-70 µM
410 with variable sensitivities dependent upon chemical structure (Carney et al., 2008); it
411 is perhaps likely that a wider range of oxygenated organic compounds produce such
412 effects. It is unclear what, if any, are the common structural characteristics of these
413 acids and more work will be needed to elucidate the mechanisms and mode(s) of
414 action (MoA). Recent work on the MoA of petroleum contaminants causing heart
415 defects in ELS of fish have suggested that individual polycyclic aromatic compounds
416 (PACs) produce a diversity of cardiotoxic mechanisms (Incardona, 2017). However,
417 most work to date has been conducted on individual aromatic hydrocarbons (Brette
418 et al., 2014). These studies have revealed multiple mechanisms linking
419 cardiomyocyte physiology to heart development, and abnormal development that
420 turn to latent impacts on physiology at later life stages (Incardona and Scholz, 2017).
421 Incardona (2017) noted that “For some PACs that are strong agonists of the aryl
422 hydrocarbon receptor (AHR), defects in heart development arise in an AHR-
423 dependent manner, which has been shown for potent organochlorine agonists, such
424 as dioxins. However, crude oil contains a much larger fraction of compounds that

425 have been found to interfere directly with cardiomyocyte physiology in an AHR-
426 independent manner". Whether the active components are the hydrocarbons or
427 common metabolites, such as the acids tested herein, remains to be better
428 elucidated. Our study indicates one potential avenue for further study.

429

430 *4.4 Relative importance of aromatic acids*

431 As deformities were found to occur following exposure to all the aromatic acids
432 tested (other than the diacid A12), and that this class of chemicals has been reported
433 to contribute about a third of the NA fraction of some OSPW (Jones et al., 2012), it
434 seems likely that such acids contribute to the developmental abnormalities observed
435 in fish exposed to OSPW (e.g. Peters et al., 2007; Marentette et al., 2015a). Of
436 course this will also depend on the concentrations of individual acids present,
437 amongst other factors. The tailings ponds of Alberta, Canada probably represent the
438 most concentrated aqueous NA but much larger volumes are generated by offshore
439 oil industries. The large dilution factors that occur when oil industry produced waters
440 are released offshore would likely reduce concentrations below those which
441 deformities were observed to occur herein, quite rapidly, but this may depend on
442 specific geographical locations. Metabolism of hydrocarbons from spilled oil (or
443 natural oil seeps) to their corresponding acids may also result in rapid dilution, and
444 reduced potential for toxic effects. However, in terrestrial oil spills this could also
445 permit mobilisation of the toxic acids through sediment pore waters leading to
446 contamination of aquifers or groundwaters. Naphthoic acids and their methyl isomers
447 were detected at concentrations of *ca*30 nM and 300 nM respectively in
448 contaminated groundwater in a former gas plant area (Testfeld Su¨d) located in

449 southwestern Germany (Annweiler et al., 2001; Safinowski et al., 2006). Naphthoic
450 and putative methylnaphthoic and dimethylnaphthoic acids plus tetralin acids were
451 also detected with estimated concentrations in the range ca5-30 nM in hydrocarbon-
452 impacted aquifers in Sedgwick County, Kansas and southwest Alberta (Safinowski et
453 al., 2006). Unlike the other acids tested in this study, the resin acid DHAA is more
454 likely to present in the environment not as a result of metabolic activity.
455 Concentrations of DHAA have been reported in wood pulp mill effluent (Makris and
456 Banerjee, 2002) similar those observed to cause deformities in embryos reported
457 herein. Overall, the environmental significance of this study is difficult to assess due
458 to the paucity of data concerned with aquatic concentrations of individual acids,
459 rather than NA mixtures. Future targeted environmental studies could now be
460 conducted based on the results presented herein.

461

462 **5. Conclusions**

463 This preliminary study demonstrates the possible toxicological importance of certain
464 aromatic petroleum acids, particularly to the ELS of fish. The experiments need to be
465 repeated for a wider range of aromatic acids with, albeit difficult, measurement of the
466 exposure concentrations in the embryonic and larval fish. The results of such studies might
467 allow focus on identification of particular individual isomeric acids in the complex NA
468 mixtures derived from degraded petroleum. Some chemicals with isobaric molecular masses
469 tested herein, demonstrated very different toxicities, indicating the importance of the
470 measurement of targeted individual pollutants, rather than mixtures.

471 Our results also show that although a commercial predictive model generally
472 produced reasonably good correspondence with experimentally derived LC_{50} values,
473 serious under- and over-estimations also occurred. Predictions may currently be

insufficiently reliable for screening purposes. The observed developmental abnormalities in zebrafish embryos exposed to aromatic NA strongly suggest that this class of chemical contributes to the reproductive toxicity reported in OSPW and certainly merits further attention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

References

Aeppli, C., Carmichael, C.A., Nelson, R.K., Lemkau, K.L., Graham, W.M., Redmond, M.C., Valentine, D.L., Reddy, C.M., 2012. Oil Weathering after the Deepwater Horizon Disaster Led to the Formation of Oxygenated Residues. Environ. Sci. Technol. 46, 8799-8807.

Agrawal, A., Gieg, L.M., 2013. In situ detection of anaerobic alkane metabolites in subsurface environments. Frontiers in Microbiology 4.

Aitken, C.M., Head, I.M., Jones, D.M., Rowland, S.J., Scarlett, A.G., West, C.E., 2018. Comprehensive two-dimensional gas chromatography-mass spectrometry of complex mixtures of anaerobic bacterial metabolites of petroleum hydrocarbons. J. Chromatogr. A 1536, 96-109.

Aitken, C.M., Jones, D.M., Larter, S.R., 2004. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature 431, 291-294.

Annweiler, E., Michaelis, W., Meckenstock, R.U., 2001. Anaerobic cometabolic conversion of benzothiophene by a sulfate-reducing enrichment culture and in a tar-oil- contaminated aquifer. Appl. Environ. Microbiol. 67, 5077-5083.

Bennett, B., Larter, S., 2000. Polar non-hydrocarbon contaminants in reservoir core extracts. Geochemical Transactions 1, 34.

Boll, M., Fuchs, G., Heider, J., 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Current Opinion in Chemical Biology 6, 604-611.

Bowman, D.T., Slater, G.F., Warren, L.A., McCarry, B.E., 2014. Identification of individual thiophene-, indane-, tetralin-, cyclohexane-, and adamantane-type carboxylic acids in composite tailings pore water from Alberta oil sands. Rapid Commun. Mass Spectrom. 28, 2075-2083.

Braunbeck, T., Bottcher, M., Hollert, H., Kosmehl, T., Lammer, E., Leist, E., Rudolf, M., Seitz, N., 2005. Towards an alternative for the acute fish LC50 test in chemical assessment: The fish embryo toxicity test goes multi-species - an update. Altex-Alternativen Zu Tierexperimenten 22, 87-102.

Brette, F., Machado, B., Cros, C., Incardona, J.P., Scholz, N.L., Block, B.A., 2014. Crude Oil Impairs Cardiac Excitation-Contraction Coupling in Fish. Science 343, 772-776.

Brown, L.D., Ulrich, A.C., 2015. Oil sands naphthenic acids: A review of properties, measurement, and treatment. Chemosphere 127, 276-290.

Carney, M.W., Erwin, K., Hardman, R., Yuen, B., Volz, D.C., Hinton, D.E., Kullman, S.W., 2008. Differential developmental toxicity of naphthoic acid isomers in medaka (Oryzias latipes) embryos. Mar. Pollut. Bull. 57, 255-266.

Clemente, J.S., Fedorak, P.M., 2005. A review of the occurrence, analyses, toxicity, and biodegradation of naphthenic acids. Chemosphere 60, 585-600.

Dissanayake, A., Scarlett, A.G., Jha, A.N., 2016. Diamondoid naphthenic acids cause in vivo genetic damage in gills and haemocytes of marine mussels. Environmental Science and Pollution Research 23, 7060-7066.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., Suflita, J.M., 2001. Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. Environ. Sci. Technol. 35, 682-689.

Frank, R.A., Fischer, K., Kavanagh, R., Burnison, B.K., Arsenault, G., Headley, J.V., Peru, K.M., Van der Kraak, G., Solomon, K.R., 2009. Effect of Carboxylic Acid Content on the Acute Toxicity of Oil Sands Naphthenic Acids. Environ. Sci. Technol. 43, 266-271.

GESAMP, 2007. Estimates of Oil Entering the Marine Environment from Sea-Based Activities; International Maritime Organization: London, 2007.

Gieg, L.M., Suflita, J.M., 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ. Sci. Technol. 36, 3755-3762.

He, Y.H., Patterson, S., Wang, N., Hecker, M., Martin, J.W., El-Din, M.G., Giesy, J.P., Wiseman, S.B., 2012. Toxicity of untreated and ozone-treated oil sands process-affected water (OSPW) to early life stages of the fathead minnow (Pimephales promelas). Water Res. 46, 6359-6368.

539 Headley, J.V., Peru, K.M., Barrow, M.P., 2015. Advances in mass spectrometric
540 characterization of naphthenic acids fraction compounds in oil sands environmental samples
541 and crude oil—a review. Mass Spectrom. Rev., n/a-n/a.
542 Incardona, J.P., 2017. Molecular Mechanisms of Crude Oil Developmental Toxicity in Fish.
543 Arch. Environ. Contam. Toxicol. 73, 19-32.
544 Incardona, J.P., Scholz, N.L., 2017. Environmental Pollution and the Fish Heart. in: Gamperl,
545 A.K., Gillis, T.E., Farrell, A.P., Brauner, C.J. (Eds.). Fish Physiology. Academic Press, pp.
546 373-433.
547 ISO, 1996. ISO 7346-1:1996 Water quality. Determination of the acute lethal toxicity of
548 substances to a freshwater fish [Brachydanio rerio Hamilton-Buchanan (Teleostei,
549 Cyprinidae)] -- Part 1: Static method. 11.
550 Jernelov, A., 2010. How to defend against future oil spills. Nature 466, 182-183.
551 Jones, D., Scarlett, A.G., West, C.E., Rowland, S.J., 2011. The toxicity of individual
552 naphthenic acids to *Vibrio fischeri*. Environ. Sci. Technol. 45, 9776-9782

553
554 Jones, D., West, C.E., Scarlett, A.G., Frank, R.A., Rowland, S.J., 2012. Isolation and
555 estimation of the 'aromatic' naphthenic acid content of an oil sands process-affected water
556 extract. J. Chromatogr. A 1247, 171-175.
557 Kannel, P.R., Gan, T.Y., 2012. Naphthenic acids degradation and toxicity mitigation in
558 tailings wastewater systems and aquatic environments: A review. J. Environ. Sci. Health Part
559 A-Toxic/Hazard. Subst. Environ. Eng. 47, 1-21.
560 Kimes, N.E., Callaghan, A.V., Aktas, D.F., Smith, W.L., Sunner, J., Golding, B.T.,
561 Drozdowska, M., Hazen, T.C., Suflita, J.M., Morris, P.J., 2013. Metagenomic analysis and
562 metabolite profiling of deep-sea sediments from the Gulf of Mexico following the Deepwater
563 Horizon oil spill. Frontiers in Microbiology 4.
564 Knag, A.C., Verhaegen, S., Ropstad, E., Mayer, I., Meier, S., 2013. Effects of polar oil
565 related hydrocarbons on steroidogenesis in vitro in H295R cells. Chemosphere 92, 106-115.
566 Lengger, S.K., Scarlett, A.G., West, C.E., Rowland, S.J., 2013. Diamondoid diacids ('O4'
567 species) in oil sands process-affected water. Rapid Commun. Mass Spectrom. 27, 2648-
568 2654.
569 Mahaffey, A., Dube, M., 2017. Review of the composition and toxicity of oil sands process-
570 affected water. Environmental Reviews 25, 97-114.
571 Makris, S.P., Banerjee, S., 2002. Fate of resin acids in pulp mill secondary treatment
572 systems. Water Res. 36, 2878-2882.
573 Malmquist, L.M.V., Christensen, J.H., Selck, H., 2013. Effects of Nereis diversicolor on the
574 Transformation of 1-Methylpyrene and Pyrene: Transformation Efficiency and Identification
575 of Phase I and II Products. Environ. Sci. Technol. 47, 5383-5392.
576 Malmquist, L.M.V., Selck, H., Jorgensen, K.B., Christensen, J.H., 2015. Polycyclic Aromatic
577 Acids Are Primary Metabolites of Alkyl-PAHs-A Case Study with Nereis diversicolor. Environ.
578 Sci. Technol. 49, 5713-5721.
579 Marentette, J.R., Frank, R.A., Bartlett, A.J., Gillis, P.L., Hewitt, L.M., Peru, K.M., Headley,
580 J.V., Brunswick, P., Shang, D., Parrott, J.L., 2015a. Toxicity of naphthenic acid fraction
581 components extracted from fresh and aged oil sands process-affected waters, and
582 commercial naphthenic acid mixtures, to fathead minnow (Pimephales promelas) embryos.
583 Aquat. Toxicol. 164, 108-117.
584 Marentette, J.R., Frank, R.A., Hewitt, L.M., Gillis, P.L., Bartlett, A.J., Brunswick, P., Shang,
585 D., Parrott, J.L., 2015b. Sensitivity of walleye (Sander vitreus) and fathead minnow
586 (Pimephales promelas) early-life stages to naphthenic acid fraction components extracted
587 from fresh oil sands process-affected waters. Environ. Pollut. 207, 59-67.
588 Melbye, A.G., Brakstad, O.G., Hokstad, J.N., Gregersen, I.K., Hansen, B.H., Booth, A.M.,
589 Rowland, S.J., Tollefsen, K.E., 2009. Chemical and toxicological characterization of an
590 unresolved complex mixture-rich biodegraded crude oil. Environ. Toxicol. Chem. 28, 1815-
591 1824.

592 NRC, 2003. Oil in the Sea III: Inputs, Fates and Effects. National Research Council (US).
593 National Academies Press, Washington, DC, p. 280.
594 Nyakas, A., Han, J., Peru, K.M., Headley, J.V., Borchers, C.H., 2013. The Comprehensive
595 Analysis of Oil Sands Processed Water by Direct-Infusion Fourier-Transform Ion Cyclotron
596 Resonance Mass Spectrometry With and Without Offline UHPLC Sample Prefractionation.
597 Environ. Sci. Technol.
598 Peters, L.E., MacKinnon, M., Van Meer, T., van den Heuvel, M.R., Dixon, D.G., 2007.
599 Effects of oil sands process-affected waters and naphthenic acids on yellow perch (Perca
600 flavescens) and Japanese medaka (Orizias latipes) embryonic development. Chemosphere
601 67, 2177-2183.
602 Petersen, K., Hultman, M.T., Rowland, S.J., Tollefsen, K.E., 2017. Toxicity of organic
603 compounds from unresolved complex mixtures (UCMs) to primary fish hepatocytes. Aquat.
604 Toxicol. 190, 150-161.
605 Reddy, C.M., Arey, J.S., Seewald, J.S., Sylva, S.P., Lemkau, K.L., Nelson, R.K., Carmichael,
606 C.A., McIntyre, C.P., Fenwick, J., Ventura, G.T., Van Mooy, B.A.S., Camilli, R., 2012.
607 Composition and fate of gas and oil released to the water column during the Deepwater
608 Horizon oil spill. Proc. Natl. Acad. Sci. U. S. A. 109, 20229-20234.
609 Reinardy, H.C., Scarlett, A.G., Henry, T.B., West, C.E., Hewitt, L.M., Frank, R.A., Rowland,
610 S.J., 2013. Aromatic Naphthenic Acids in Oil Sands Process-Affected Water, Resolved by
611 GCxGC-MS, Only Weakly Induce the Gene for Vitellogenin Production in Zebrafish (Danio
612 rerio) Larvae. Environ. Sci. Technol. 47, 6614-6620.
613 Rissanen, E., Krumschnabel, G., Nikinmaa, M., 2003. Dehydroabietic acid, a major
614 component of wood industry effluents, interferes with cellular energetics in rainbow trout
615 hepatocytes. Aquat. Toxicol. 62, 45-53.
616 Rowland, S.J., Clough, R., West, C.E., Scarlett, A.G., Jones, D., Thompson, S., 2011a.
617 Synthesis and mass spectrometry of some tri-and tetracyclic naphthenic acids. Rapid
618 Commun. Mass Spectrom. 25, 2573-2578.
619 Rowland, S.J., Scarlett, A., West, C., Jones, D., Frank, R., 2011b. Diamonds in the rough:
620 identification of individual naphthenic acids in oil sands process water. Environ. Sci. Technol.
621 45, 3154-3159.
622 Rowland, S.J., West, C.E., Jones, D., Scarlett, A.G., Frank, R.A., Hewitt, L.M., 2011c.
623 Steroidal Aromatic 'Naphthenic Acids' in Oil Sands Process-Affected Water: Structural
624 Comparisons with Environmental Estrogens. Environ. Sci. Technol. 45, 9806–9815.
625 Ruddy, B.M., Huettel, M., Kostka, J.E., Lobodin, V.V., Bythell, B.J., McKenna, A.M., Aeppli,
626 C., Reddy, C.M., Nelson, R.K., Marshall, A.G., Rodgers, R.P., 2014. Targeted Petroleomics:
627 Analytical Investigation of Macondo Well Oil Oxidation Products from Pensacola Beach.
628 Energy Fuels 28, 4043-4050.
629 Safinowski, M., Griebler, C., Meckenstock, R.U., 2006. Anaerobic cometabolic
630 transformation of polycyclic and heterocyclic aromatic hydrocarbons: Evidence from
631 laboratory and field studies. Environ. Sci. Technol. 40, 4165-4173.
632 Scarlett, A.G., Reinardy, H.C., Henry, T.B., West, C.E., Frank, R.A., Hewitt, L.M., Rowland,
633 S.J., 2013. Acute toxicity of aromatic and non-aromatic fractions of naphthenic acids
634 extracted from oil sands process-affected water to larval zebrafish. Chemosphere 93, 415-
635 420.
636 Scarlett, A.G., West, C.E., Jones, D., Galloway, T.S., Rowland, S.J., 2012. Predicted toxicity
637 of naphthenic acids present in oil sands process-affected waters to a range of environmental
638 and human endpoints. Sci. Total Environ. 425, 119-127.
639 Sepulveda, M.S., Quinn, B.P., Denslow, N.D., Holm, S.E., Gross, T.S., 2003. Effects of pulp
640 and paper mill effluents on reproductive success of largemouth bass. Environ. Toxicol.
641 Chem. 22, 205-213.
642 Sorensen, L., Sorhus, E., Nordtug, T., Incardona, J.P., Linbo, T.L., Giovanetti, L., Karlsen, O.,
643 Meier, S., 2017. Oil droplet fouling and differential toxicokinetics of polycyclic aromatic
644 hydrocarbons in embryos of Atlantic haddock and cod. Plos One 12.

Straus, D., Stuthridge, T., Anderson, S., Gifford, J., 1997. Acute toxicity of dehydroabietic acid to rainbow trout: manipulation of biotransformation enzymes. Australasian Journal of Ecotoxicology 3, 131-139.
Thomas, K.V., Langford, K., Petersen, K., Smith, A.J., Tollefsen, K.E., 2009. Effect-Directed Identification of Naphthenic Acids As Important in Vitro Xeno-Estrogens and Anti-Androgens in North Sea Offshore Produced Water Discharges. Environ. Sci. Technol. 43, 8066-8071.
Tollefsen, K.E., Petersen, K., Rowland, S.J., 2012. Toxicity of Synthetic Naphthenic Acids and Mixtures of These to Fish Liver Cells. Environ. Sci. Technol. 46, 5143-5150.
Valentine, D.L., Fisher, G.B., Bagby, S.C., Nelson, R.K., Reddy, C.M., Sylva, S.P., Woo, M.A., 2014. Fallout plume of submerged oil from Deepwater Horizon. Proc. Natl. Acad. Sci. U. S. A. 111, 15906-15911.
Wan, Y., Wang, B., Khim, J.S., Hong, S., Shim, W.J., Hu, J., 2014. Naphthenic Acids in Coastal Sediments after the Hebei Spirit Oil Spill: A Potential Indicator for Oil Contamination. Environ. Sci. Technol. 48, 4153-4162.
West, C.E., Pureveen, J., Scarlett, A.G., Lengger, S.K., Wilde, M.J., Korndorffer, F., Tegelaar, E.W., Rowland, S.J., 2014. Can two-dimensional gas chromatography/mass spectrometric identification of bicyclic aromatic acids in petroleum fractions help to reveal further details of aromatic hydrocarbon biotransformation pathways? Rapid Commun. Mass Spectrom. 28, 1023-1032.
White, H.K., Xu, L., Hartmann, P., Quinn, J.G., Reddy, C.M., 2013. Unresolved Complex Mixture (UCM) in Coastal Environments Is Derived from Fossil Sources. Environ. Sci. Technol. 47, 726-731.
Wilde, M.J., Rowland, S.J., 2015. Structural identification of petroleum acids by conversion to hydrocarbons and multi-dimensional gas chromatography-mass spectrometry. Anal. Chem.
Wilde, M.J., Rowland, S.J., 2018. Naphthenic acids in oil sands process waters: Identification by conversion of the acids or esters to hydrocarbons. Org. Geochem. 115, 188-196.
Wilde, M.J., West, C.E., Scarlett, A.G., Jones, D., Frank, R.A., Hewitt, L.M., Rowland, S.J., 2015. Bicyclic naphthenic acids in oil sands process water: Identification by comprehensive multidimensional gas chromatography–mass spectrometry. J. Chromatogr. A 1378, 74-87.

678 Table and Figure legends

679

680 Table 1 Physical and predicted properties of test chemicals

681 Table 2 Predicted and measured lethal toxicities of test chemicals to invertebrates
682 and fish

683

684 Figure 1 Concentration response relationships for *D. rerio* exposed to aromatic
685 carboxylic acids. Codes, top left of each panel refer to chemical names provided in
686 Table 1 and structures in Table 2. Curves are predicted by Probit analyses. Error
687 bars for observed mortality are standard errors of the mean.

688 Figure 2 *D. rerio* embryos 72 h post fertilisation exposed to (A) 0 μM, (B) 0.8 μM (C)
689 1.5 μM, (D) 3.0 μM, (E) 6.1 μM and (F) 12.2 μM 1-pyrene carboxylic acid.
690 Abnormalities was included yolk-sac edema, fin abnormalities, pericardial edema, tail
691 flexure and truncation and stunted growth. Arrows indicate (1) yolk-sac and (2)
692 pericardial edema

693 Figure 3 Arrows indicate head and ocular edemas in *D. rerio* embryos exposed to
694 25.5 μM naphthalene-2-carboxylic acid.

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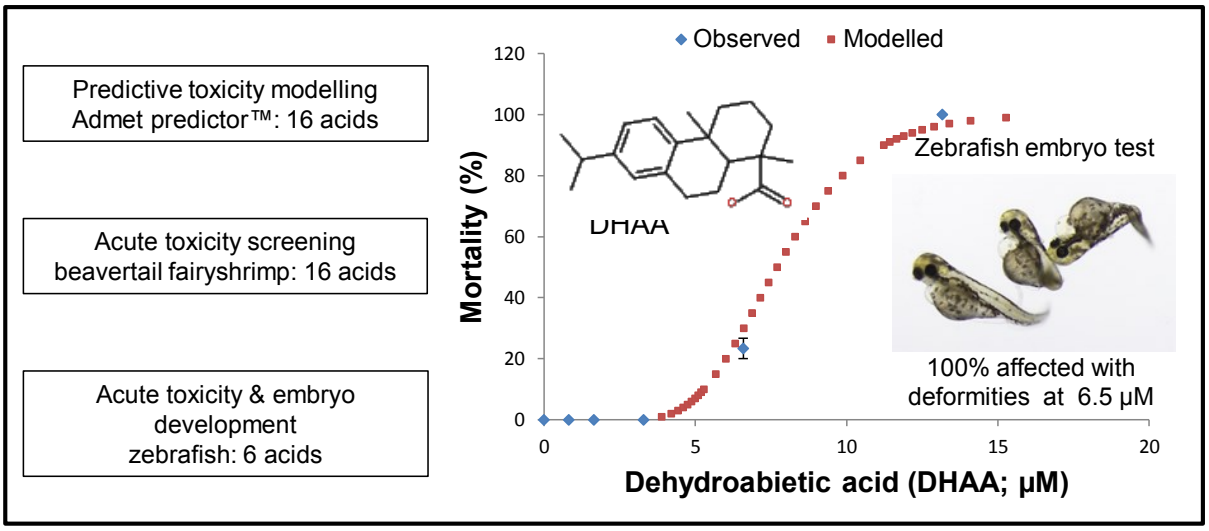
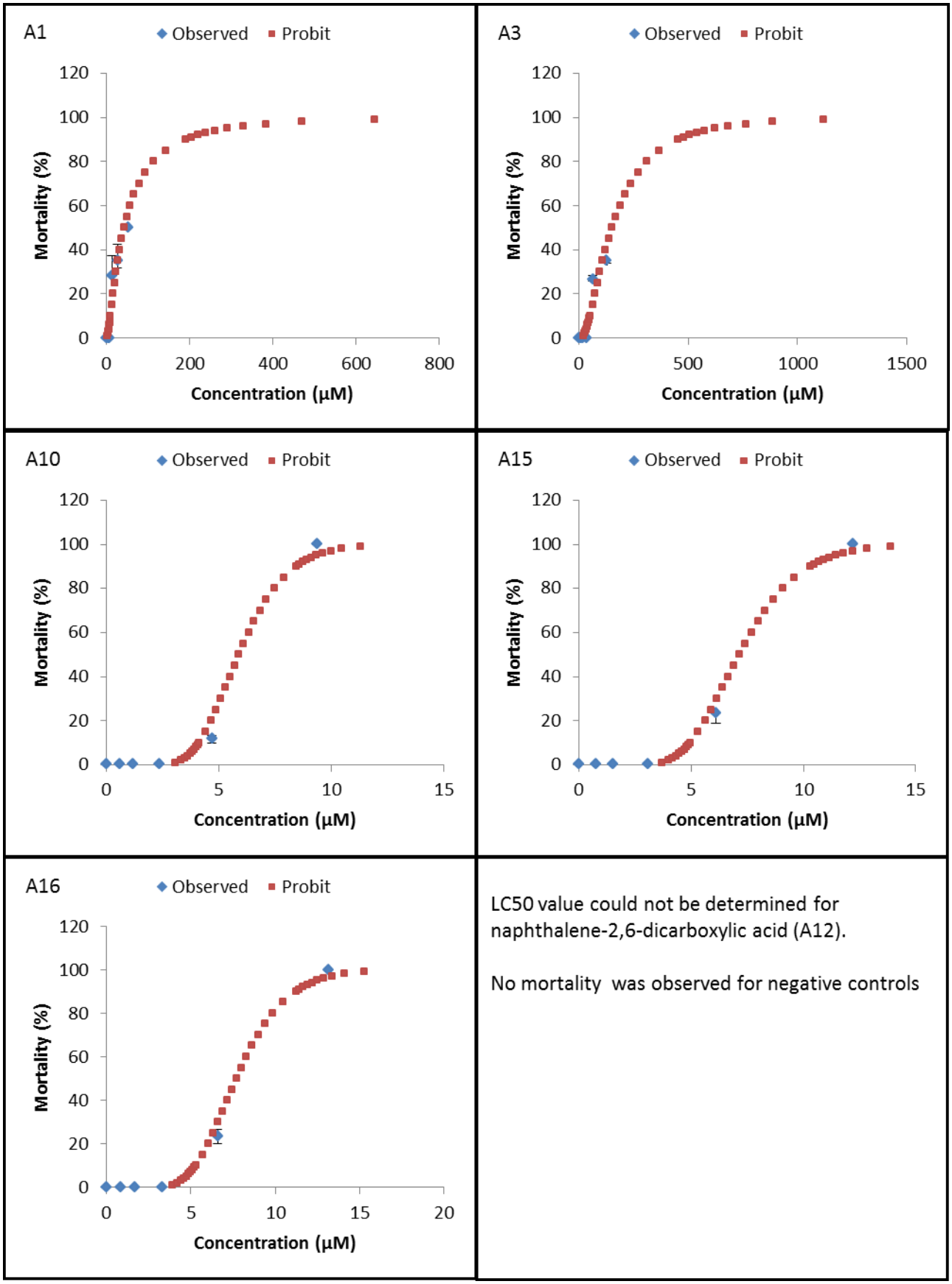


697

698 Graphical abstract



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700 Fig. 1

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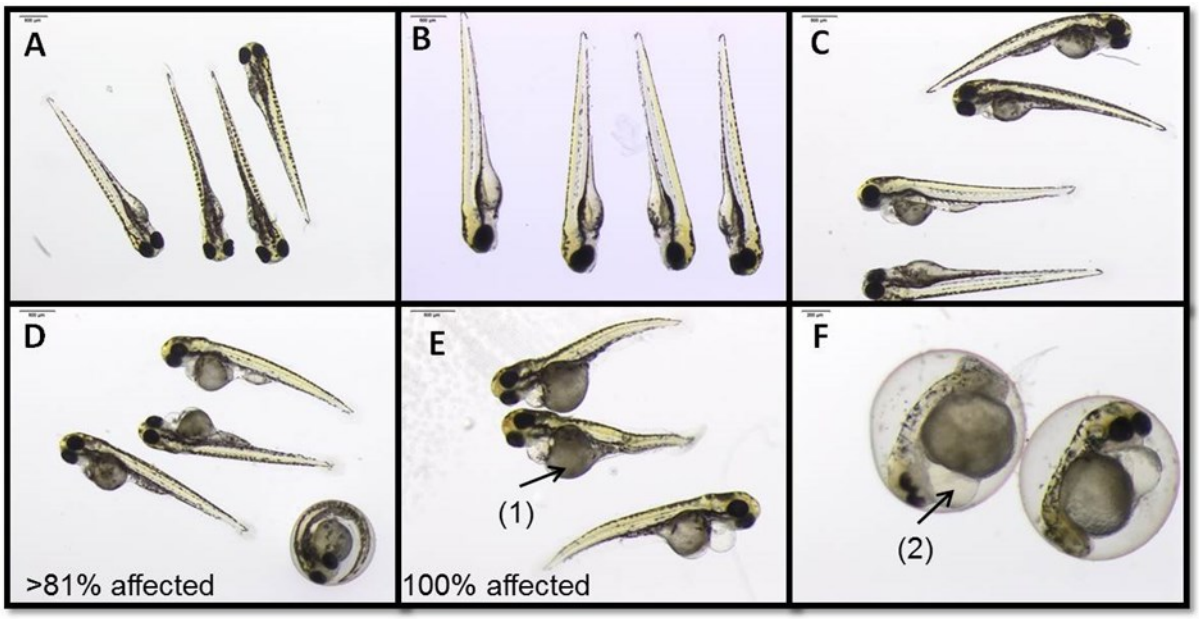
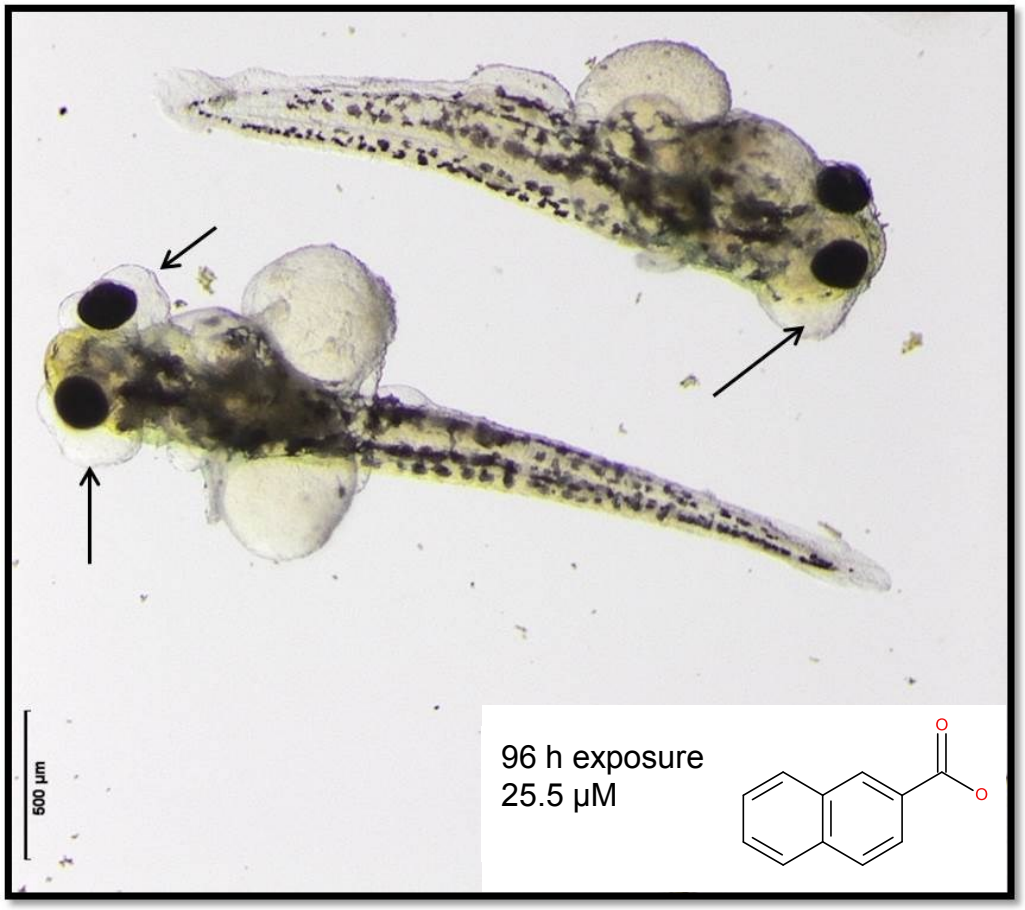


Fig. 2



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707 Figure 3